(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
22 February 2001 (22.02.2001)

PCT

(10) International Publication Number
WO 01/12828 A1

(51) International Patent Classification[7]: C12N 15/82, A01H 1/00

(21) International Application Number: PCT/US00/22565

(22) International Filing Date: 17 August 2000 (17.08.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/149,582 18 August 1999 (18.08.1999) US

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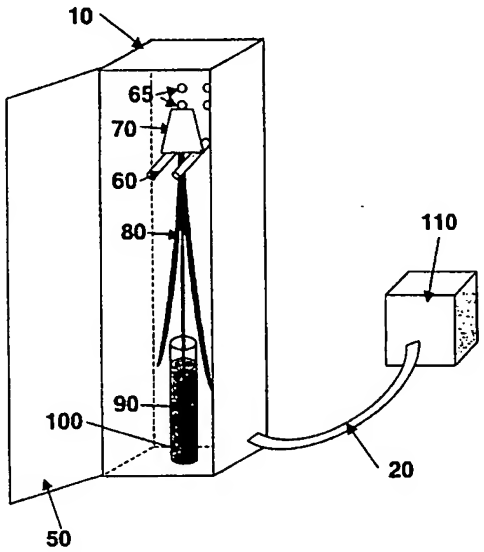
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHODS AND APPARATUS FOR TRANSFORMATION OF MONOCOTYLEDENOUS PLANTS USING *AGROBACTERIUM* IN COMBINATION WITH VACUUM FILTRATION



(57) Abstract: A method for transforming monocotyledenous plants such as rice, wheat, rice, corn, barley, sorghum, millet, teff, rye, and oats involves contacting the flowers of the monocotyledenous plant with an *Agrobacterium* solution or suspension, and subjecting the plant to a vacuum such that the *Agrobacterium* enters the flowers of the plant. The method is useful in the generation of transgenic monocotyledenous plants. The method is preferably carried out in a vacuum chamber, which forms part of an apparatus useful in the practice of the invention.

WO 01/12828 A1

METHODS AND APPARATUS FOR TRANSFORMATION OF MONOCOTYLEDENOUS PLANTS USING *AGROBACTERIUM* IN COMBINATION WITH VACUUM FILTRATION

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application 60/149,582 filed August 18, 1999.

Field of The Invention

This invention relates to the transformation of monocotyledenous plants, and especially the transformation of rice plants.

Background of the Invention

One of the primary goals of plant genetic research and development is the production of transgenic plants that express a heterologous gene (*i.e.*, produce a protein from a gene that normally does not occur in the transgenic plant) in an amount sufficient to confer a desired phenotype to the plant. While significant advances have been made in pursuit of this goal, the transformation of heterologous genes into certain plants and the expression of these genes in the plants remains problematic.

In general, plant transformation refers to stably introducing a nucleic acid segment carrying a functional gene (generally, a heterologous gene) into a plant that did not previously contain that gene. In a successful transformation, a DNA construct containing a structural coding sequence is inserted into the genome of a plant by one of several known methods. Examples of known transformation methods include direct gene transfer into protoplasts, microprojectile bombardment, injection into protoplasts, cultured cells and tissues or meristematic tissues, electroporation, and *Agrobacterium*-mediated transformation.

Agrobacterium-mediated gene transfer exploits the natural ability of *A. tumefaciens* and *A. rhizogenes* to transfer DNA into plant chromosomes. *Agrobacterium* is a plant pathogen that transfers a set of genes encoded in a region called T-DNA of the Ti and Ri plasmids of *A. tumefaciens* and *A. rhizogenes*, respectively, into plant cells. Transformation using *A. rhizogenes* developed

analogously to that of *A. tumefaciens* and has been successfully utilized to transform, for example, alfalfa, *Solanum nigrum L.*, and poplar. *See* U.S. Patent No. 5,777,200 to Ryals et al., the disclosure of which is incorporated herein by reference in its entirety. The typical result of transfer of the Ti plasmid is a tumorous growth called a crown gall in which the T-DNA is stably integrated into a host chromosome. Integration of the Ri plasmid into the host chromosomal DNA results in a condition known as "hairy root disease." The ability to cause disease in the host plant can be removed by deletion of the genes in the T-DNA without loss of DNA transfer and integration. The DNA to be transferred (*i.e.*, the heterologous gene) is attached to border sequences that define the end points of an integrated T-DNA.

Gene transfer by means of engineered *Agrobacterium* strains has become routine for many dicotyledonous crop plants. Considerable difficulty has been experienced, however, in using *Agrobacterium* to transform monocotyledonous plants (monocots), and in particular, cereal plants such as rice. Several attempts to use *Agrobacterium* to transform monocots have focused on wounding certain plant tissues in order to facilitate entry of the *Agrobacterium* into the plant cells. Few if any of these attempts have met with the successful regeneration of transgenic plants from the transformed cells. U.S. Patent No. 5,187,073 to Goldman et al. describes a process of transforming plants of the family *Gramineae* (*e.g.*, corn, wheat, barley, rye and oats) using an *Agrobacterium* transformation system, wherein a graminaceous seedling is wounded in an area containing rapidly dividing cells (*i.e.*, the apical meristem), after which the wound is inoculated with *Agrobacterium*. J. Gould et al., *Plant Physiol.* **95**, 426-434, describes the use of supervirulent *Agrobacterium* vectors in transforming injured (*i.e.*, wounded) apices in maize. P.A. Mooney et al., *Plant, Cell, Tissue, Organ Culture* **25**, 209-218 (1991), describes the attempt to introduce the kanamycin resistance gene into wheat embryos. The embryos were wounded using an enzyme, then inoculated with *Agrobacterium*. Although a small number of calli assumed to be transformants grew, no whole plants were able to be generated. Similarly, Raineri *et al.*, *Bio/Technol.* **8**, 33-38 (1990) inoculated a supervirulent strain of *Agrobacterium* into eight varieties of rice after injuring the scutella of rice plants. Although certain resistant calli were observed, transformed plants could not be obtained from the calli.

Bechthold and Bouchez (Gene Transfer to Plants, Potrykus et al., eds., Springer-Verlag Berlin, 1995, p. 19), and Bechtold and Pelletier (Methods Mol Biol. 1998;82:259-66) describe vacuum infiltration of Arabidopsis thaliana plants for production of transgenic seeds. However, no other crops are mentioned and the authors speculate that certain aspects of Arabidopsis thaliana, like size of the plant, cycle length, and reproductive biology might prevent this transformation procedure from being applied to many other plant species.

U.S. Patent No. 5,591,616 and European Patent Application 0 897 013 A1, both to Hiei, describe methods of transforming monocots such as rice with an *Agrobacterium* system, including so-called "super-binary vectors." These methods do not require the wounding of the monocot plant tissue; however, the methods described therein do require the use of prepared "de-differentiated cultured tissue" such as callus, or adventitious embryo-like tissue in cell culture. The use of whole rice plants is not described therein.

U.S. Patent No. 5,611,172 to Dugan et al. describes an apparatus for the treatment of a live plant comprising a sealed pressure vessel, a vacuum system, and a root treatment system. The pressure vessel is used to receive a container having a rooting medium and the roots of the live plant contained therein. The system is used to remove a predetermined amount of fluid from the rooting medium, and then add a certain amount of root treatment solution to the rooting medium. The reference does not describe the use of a vacuum system for the transformation of plants, and discusses the treatment of plant roots only.

U.S. Patent No. 5,994,624 to Trolinder et al. describes the injection of Agrobacteria suspensions into plants and plant tissue using a needleless high-pressure injection device. This work aims at the transformation of germline cells of the plant that develop into seeds. However, the reference does not use vacuum infiltration to apply the Agrobacteria to the floral tissue. Instead, injection of the suspension through several cell layers can damage the plant and therefore interfere with normal seed development.

U.S. Patent No. 6,037,522 to Dong et al. describes a method for Agrobacterium-mediated transformation of monocots. Monocot inflorescences are dissected from the plants and cultured in callus-inducing tissue culture media, where the inflorescences, or the callus derived thereof, is co-cultivated with the

Agrobacterium cells. This method therefore includes tissue culture steps and is not an *in planta* transformation method.

In light of the foregoing, it would be desirable to have a means for reliably and quickly transforming monocots using *Agrobacterium* mediated transformations in which whole plants may conveniently be used, thus avoiding the time-consuming and sometimes difficult processes of dissecting selected plant tissues and cocultivating the dissected parts with growth media, generating cell cultures, calli and the like.

Summary of the Invention

Certain objects, advantages and novel features of the invention will be set forth in the description that follows, and will become apparent to those skilled in the art upon examination of the following, or may be learned with the practice of the invention.

It is an object of the present invention to provide a method for efficiently, quickly and reliably transforming monocotyledenous plants such as rice plants with heterologous genes using *Agrobacterium* transformation systems.

It is a further object of the present invention to be able to generate transgenic monocot plants.

It is yet an additional object of the present invention to provide apparatus that are useful in the transformation of monocots.

Accordingly, one aspect of the present invention is directed toward a method of transforming a monocotyledenous plant, comprising contacting at least one flower of the monocot plant with a solution or suspension comprising at least one or more *Agrobacterium* clone(s), and subjecting the flower to a vacuum effective to cause the *Agrobacterium* clone to enter the flower(s) of the plant (*i.e.*, so that T-DNA from Agrobacteria can be inserted into the germline cells of the plant). The method is preferably carried out in a vacuum chamber. In one preferred embodiment of the invention, the monocot plant is affixed in a vacuum chamber such that the flowers of the rice plant are in contact with the *Agrobacterium* solution, the solution being contained within the chamber. A vacuum is then generated in the vacuum chamber while the flowers of the plant are in contact with the *Agrobacterium* solution.

In a preferred embodiment of the invention, the monocot is a rice plant, and the *Agrobacterium* vector comprises one or more heterologous genes. Accordingly, the present invention finds use in producing transgenic plants such as transgenic rice plants.

Apparatus that are useful in the practice of the present invention generally and preferably comprise a vacuum chamber, means for generating a vacuum, and a connector between the vacuum generating means and the vacuum chamber. The apparatus also comprises means for affixing or supporting a plant inside the vacuum chamber.

The methods and apparatus of the present invention provide several advantages over existing methods of monocot transformation, and especially over existing methods of rice transformation. The present invention also advantageously utilizes whole plants, thus relieving the practitioner of the need to prepare cell cultures or samples of specialized plant tissues such as calli or protoplasts or embryonic tissue. Further, the present invention does not require the wounding of the plants for successful transformation.

The foregoing and other aspects of the present invention are explained in detail in the specification set forth below.

Brief Description of the Drawings

Fig. 1 is a schematic illustration of an embodiment of the present invention.

Detailed Description of the Preferred Embodiments

The present invention will now be described more fully hereinafter with reference to the accompanying drawing, in which a preferred embodiment of the invention is shown. This invention may, however, be embodied in different forms and should not be construed as limited to the embodiments set forth herein. Rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art. Like numbers refer to like elements throughout.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

The present invention finds use in the transformation of monocotyledonous plants with *Agrobacterium* transformation systems. As used herein, the term "transformation" refers to the stable introduction of a nucleic acid sequence or polynucleotide of DNA into a plant, plant tissue or plant cell. Suitable monocots include, but are not limited to, all monocots, particularly those plants in the grass family *Poaceae*, including but not limited to wheat, rice, corn, barley, sorghum, millet, teff, rye, and oats, with rice being particularly preferred. Monocots of the families *Palmaceae*, *Araceaea*, *Musaceaea*, and *Liliaceae* are also suitable subjects for transformation by the methods and apparatus of the present invention.

As provided above, rice plants are preferred in the practice of the present invention. Any *Oryza* (rice) variety is suitable for transformation according to the methods of the present invention, with *Oryza sativa* being preferred. Within the species *O. sativa*, Japonica and Indica varieties are preferred.

"Agrobacterium clone" refers to Agrobacterium cells harboring or containing a vector comprising one or more T-DNA(s) for transforming a monocotyledonous plant.

"DNA" refers to deoxyribonucleic acid. "DNA" is considered to be substantially the same as "DNA sequence" or "DNA polynucleotide" or "polynucleotide" and may be used interchangeably.

The terms "flowers" and "inflorescence" are substantially equivalent and may be used interchangeably. Generally, an inflorescence is comprised of a cluster or clusters of flowers. In rice, the inflorescence is known as a panicle. Since the method of the present invention utilize whole or entire plants, the flower(s) or inflorescence remains attached to the plant as part of its normal growth and development.

"*In planta*" refers to methods of the present invention which use the whole or entire plant. Such *in planta* methods are distinctly different from *in vitro* methods which use isolated tissues or cells of a plant, especially in conjunction or co-cultivation with growth medium or media for dissection of plant tissues and plant cells.

"Monocots" or "monocotyledonous" refers to plants having one cotyledon such as rice, maize (corn), wheat, sorghum and the like.

"Strain" refers to a stock of Agrobacterium cells from a specific source which are maintained in successive cultures for subsequent plant treatment, contact or innoculation.

"T-DNA" refers to a DNA sequence or polynucleotide, a copy of which gets transferred from Agrobacterium to the plant cell.

"T-DNA borders" refers to the DNA sequences that flank the T-DNA.

"Transforming" or "the transformation process" introduces a DNA sequence into a recipient plant cell and its subsequent integration into the plant cell's chromosomal DNA. In addition to the DNA sequence of interest, a selectable marker gene can be placed within the T-DNA borders in order to allow selection for plants transformed with the DNA sequence of interest. Such selectable marker genes include *aph4*, for hygromycin resistance, *npt2*, for kanamycin resistance, *bar* for Basta resistance, *cp4* for glyphosate resistance.

The term "plasmid" refers to a small, independently replicating piece of DNA (i.e. exists inside a bacterial cell separate from the bacteria's main DNA).

The term "vector" refers to a plasmid that can be used to transfer DNA sequences from one organism to another. For example, the present invention uses a vector to transfer DNA from an Agrobacterium clone to a plant cell.

The present invention preferably accomplishes the transformation of monocotyledonous plants (monocots) by using entire or whole plants, rather than isolated tissues or cells of the plants. Preferably, the plants transformed in the present invention are in the flowering stage, or in the growth stage immediately prior to the flowering stage. Plants transformed by the methods of the present invention may be transformed prior to the pollination of the plant or after the pollination of the plant, with transformation prior to the pollination of the plant being preferred. It is also preferable that the monocot plants of the present invention are transformed prior to the plant's development of seeds. In the case of rice plants, the rice plants can be transformed before, during or after the growth phase known as "heading" (i.e. the stage where the panicle emerges from the plant sheath and becomes visible). In rice plants, it is preferred that the panicles of the plants are treated during or before the heading stage.

One preferred embodiment of the apparatus and methods of the present invention is shown in **FIG. 1**. A vacuum chamber **10** is connected by a vacuum connector **20** to a vacuum generator (*i.e.*, vacuum pump **110**). The vacuum

generator is preferably controllable by an operator, such that the pressure level inside the vacuum chamber **10** may be easily controlled, and the vacuum itself easily created or released. The vacuum chamber **10** may be a bell jar that is connected by a vacuum connector **20** to vacuum generating means. Preferably, the vacuum chamber **10** is a container of a material suitable and sturdy enough to withstand and contain vacuum conditions (*e.g.*, glass, plastic, or metal, selected according to those skilled in the art), and has a top, bottom, and at least three (and preferably four) walls, with one wall optionally comprising a door. Such a door, shown as element **50** in **FIG. 1** may be opened in order to place materials (*e.g.*, plants) inside the vacuum chamber **10**, and to remove materials from the vacuum chamber **10**. When closed, the door **50** may be sealed by any external means known in the art (*i.e.*, by a latch, lock, clamp, sealing adhesive, or the like, selected by one knowledgeable in the art). Alternatively, once the door **50** is closed, the vacuum chamber **10** may be sealed by the generation of a vacuum, the creation of the vacuum inside the vacuum chamber **10** being sufficient to seal the chamber. Although the door **50** is illustrated as being located at the front of the vacuum chamber **10**, one skilled in the art will appreciate that the door may be located on any surface of the vacuum chamber **10** (*i.e.*, the top, back or bottom), or may comprise only a portion of any surface of the vacuum chamber **10** (*i.e.*, may be only a portion of the top, back, bottom or top of the vacuum chamber **10**).

As shown in **FIG. 1**, a first container **70** (*i.e.*, a pot) in which a flowering or pre-flowering monocot plant **80** is growing is placed inside the vacuum chamber **10** such that the plant is inverted in the vacuum chamber **10** (*i.e.*, the container is situated towards the top of the vacuum chamber **10**, while the top of the plant **80** is directed toward the bottom of the chamber). The container **70** may contain soil, and any other nutrient, additive, or material that is necessary, useful, or desirable in the growing of the plant **80**. The container **70** is supported, affixed or held in place inside the chamber by any suitable means that will be apparent to one skilled in the art. In **FIG. 1**, the means for holding the container **70** in the vacuum chamber **10** are shown as a pair of dowels or rods **60**, which are inserted into corresponding parallel support holes/indentations **65** at the back of the vacuum chamber **10**. The dowels **60** are arranged such that they are sufficiently spaced to support the top of the container **70** when the container **70** is inverted and then placed onto the dowels **60**. If such an arrangement is used to support the container in the vacuum chamber

10, preferably the dowels **60** are removable and a series of in-line, parallel support holes/indentations **65** are located vertically along the back of the vacuum chamber **10**, as illustrated in **FIG. 1**, such that the location of the dowels **60** (and thus, the distance of the container from the bottom of the vacuum chamber **10**) may be adjusted. The skilled artisan will recognize, however, that many means of supporting the container **70** in the vacuum chamber **10** will be useful in the practice of the present invention, including suspending the container **70** in the vacuum chamber **10** by tying or binding means, placing the container **70** on a rack located within the vacuum chamber **10**, placing a magnet on the bottom of the container **70** and attaching the container **70** to the top of the vacuum chamber **10** (if the top of the vacuum chamber **10** is capable of such magnetic attachment), and the like. Test stand systems for vacuum chambers are described in U.S. Patent No. 3,714,833 to Newman (the disclosure of which is incorporated by reference herein in its entirety), and are also suitable in the practice of the present invention. The method and/or means of supporting or affixing the first container **70** in the vacuum chamber **10** is not critical to practice of the invention.

Vacuum chambers **10** useful in the practice of the present invention are commercially available from suppliers and manufacturers. Alternatively, the skilled artisan may create an apparatus useful in the present invention, including the vacuum chamber **10**, according to the guidance provided herein.

Preferably, the vacuum chamber **10** is sufficiently sized to hold at least one container **70** in which is growing a flowering or pre-flowering monocot plant **80**. Although **FIG. 1** is illustrated to show the use of a chamber **10** in which only one container **70** is placed within, the skilled artisan will appreciate that another embodiment of the invention will be a vacuum chamber **10** large enough to include more than one container **70**, or large enough to hold one container **70** that contains more than one monocot plant **80**.

Although reference is made herein to a plant growing in soil/nutrients in a container (*e.g.*, a pot), one skilled in the art will recognize that the presence of the container is not critical to the practice of the invention. Rather, what is desired is that the plant have contact with (*i.e.*, grow in) the nutrients necessary and/or desirable for growth both before the plant is transformed and after the plant has been transformed, thereby allowing the plant to mature and produce seeds. Therefore, if the nutrients needed by a growing plant are provided, for example,

hydroponically, then the plant may be temporarily removed from the hydroponic environment in order to perform the transformation as described herein, and then returned to the hydroponic environment after transformation. In such an embodiment of the invention, the plant **80** itself is suspended inside the vacuum chamber **10** such that the plant **80** is inverted in the vacuum chamber **10** (*i.e.*, the roots of the plant **80** are situated towards the top of the vacuum chamber **10**, while the top of the plant **80** is directed toward the bottom of the vacuum chamber **10**). The plant **80** may be suspended in the vacuum chamber **10** by any means that will be readily apparent to one skilled in the art.

In an alternative embodiment of the invention, the entire monocot plant **80** is not present in the vacuum chamber **10**. In this embodiment, while the entire plant **80** remains intact, only the flowers or flowering portion of the plant **80** are present in the vacuum chamber **10**, while the rest of the plant (*e.g.*, the roots and stem) are outside the vacuum chamber **10**. Those skilled in the art will appreciate that such a configuration may be achieved with a vacuum chamber **10** that has an opening (*i.e.*, a collared aperture) into which the flowering part of the plant **80** may be inserted, such that the vacuum chamber **10** contains only the flowering part of the plant **80**, while the other parts of the plant remain outside the vacuum chamber **10**. In this alternative embodiment, the flowering part of the plant **80** within the vacuum chamber **10** is sealed off from the rest of the plant (*i.e.*, by the closing or sealing of a collared aperture in the vacuum chamber **10**) when the flowers of the plant **80** are subjected to vacuum treatment as described herein, such that the flowers of the plant **80** are subjected to vacuum treatment but the rest of the plant **80** is not subjected to the vacuum treatment.

Referring again to **FIG. 1**, the vacuum chamber **10** also contains a second container **100** in which is contained a solution or suspension comprising *Agrobacterium* (hereinafter referred to as the "*Agrobacterium* solution") which solution is described in further detail below. The second container **100** may be any suitable container recognized in the art as useful for holding a solution, including but not limited to a test tube, a beaker, flask, plate, bowl, or the like.

As provided above, the plant **80** growing in container **70** is positioned in the vacuum chamber **10** such that the top of the plant **80** is pointed in the direction of the bottom of the vacuum chamber **10**. If the monocot plant **80** is a rice plant, it is preferred that the panicle **90** of the plant **80** is visible. Optionally and preferably,

the leaf sheath that normally covers the panicle **90** is temporarily removed from the panicle **90** of the plant **80**. As shown in **FIG. 1**, the first container **70** is supported in the vacuum chamber **10** such that the panicles **90** (and, in the case of other flowering or pre-flowering monocots, the flowers) of the monocot plant **80** are in contact with, and preferably submerged or immersed in the *Agrobacterium* solution contained within the second container **100**.

The "*Agrobacterium* solution or suspension" of the present invention comprises at least one clone of *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*, preferably *Agrobacterium tumefaciens*, in a liquid or suspension medium. Such media are well known in the art. Suitable media for transforming plants with Agrobacterium are known in the art and disclosed for example by Hiei et al. (1994; Plant J. 6:271). Preferred media for use which may be used with the method of the present invention are described by Bechtold et al. (1993; Life Sci 316: 1194) and Bent et al. (1994; Science 265:1856), the disclosures of which are incorporated herein by reference. For high-throughput procedures, preferably the methodology described in U.S. Patent Application Serial No. 09/607306 filed 30 June 2000, titled "Improved Method for Transforming Plants." The methodology described in this patent application employs a diluted suspension of Agrobacterium, thus avoiding the need to pellet the Agrobacterium using centrifuges. In a preferred embodiment of the present invention, the *Agrobacterium* solution or suspension comprises an *Agrobacterium* binary vector. The *Agrobacterium* solution or suspension of the present invention may optionally and preferably also contain additional agents such as wetting/accession agents or surfactants (*i.e.*, Silwet L-77, pluronic F-68 (Sigma), and the like) that are known to enhance transformation. The *Agrobacterium* solution or suspension employed in the present invention may optionally and preferably also contain one or more agent which can induce (i.e. activate) the vir region genes. Such agents include acetosyringone and other phenolic compounds like alpha-hydroxy-acetosyringone or sugars like D-glucose and non-catabolizable sugars such as 2-deoxy-D-glucose and 6-deoxy-D-glucose.

A variety of *Agrobacterium* strains are known in the art and may be used in the methods of the invention. *See, e.g.*, Hooykaas, *Plant Mol. Biol.* **13**, 327 (1989); Smith et al., *Crop Science* **35**, 301 (1995); Chilton, *Proc. Natl. Acad. Sci. USA* **90**, 3119 (1993); Mollony et al., *Monograph Theor. Appl. Genet. NY* **19**, 148 (1993);

Ishida et al., *Nature Biotechnol.* **14**, 745 (1996); and Komari et al., *The Plant Journal* **10**, 165 (1996), the disclosures of which are incorporated herein by reference in their entirety.

Agrobacterium clones of the present invention are preferably maintained on *Agrobacterium* master plates with stock frozen at about -80°C. Master plates can be used to inoculate agar plates to obtain *Agrobacterium* that is then resuspended in medium for use in the infection process. Alternatively, bacteria from the master plate can be used to inoculate broth cultures that are grown to logarithmic phase prior to transformation.

Any suitable *Agrobacterium* clones for transforming the monocot may be employed according to the present invention. As provided above, *Agrobacterium* is a plant pathogen that transfers a set of genes encoded in a region called T-DNA of the Ti and Ri plasmids of *A. tumefaciens* and *A. rhizogenes*, respectively, into plant cells. In addition to the T-region, the Ti (or Ri) plasmid contains a vir region. The vir region is important for efficient transformation, and appears to be species-specific. Binary vector systems have been developed where the manipulated disarmed T-DNA carrying, for example, heterologous DNA and the vir functions are present on separate plasmids. In other words, a heterologous nucleic acid sequence (*i.e.*, gene or genes) of interest and the flanking T-DNA can be carried by a binary vector lacking the vir region. The vir region is then provided on a disarmed Ti- plasmid or on a second binary plasmid. In this manner, a modified T-DNA region comprising heterologous DNA is constructed in a small plasmid which replicates in *E. coli*. This plasmid is transferred conjugatively in a tri-parental mating or via electroporation into *A. tumefaciens* that contains a compatible plasmid with virulence gene sequences. The vir functions are supplied in trans to transfer the T-DNA into the plant genome. As another alternative, the heterologous nucleic acid sequence and the T-DNA border sequences can be put into the T-DNA site on the Ti-plasmid through a double recombination event by which the new T-DNA replaces the original Ti-plasmid T-DNA. The vir region can be supplied by the Ti-plasmid or on a binary plasmid. As yet a further alternative, the heterologous nucleic acid sequence and flanking T-DNA can be integrated into the bacterial chromosome as described by U.S. Patent No. 4,940,838 to Schilperoort et al., and the vir region can then be supplied on a Ti-

plasmid or on a binary plasmid. Binary vectors as described herein are useful in the practice of the present invention, and are preferred.

Alternately, in other embodiments of the invention, super-binary or "supervirulent" *Agrobacterium* vectors are employed in the *Agrobacterium* solutions or suspensions. *See, e.g.*, U.S. Patent No. 5,591,615 and EP 0 604 662, herein incorporated by reference. Such a super-binary vector has been constructed containing a DNA region originating from the hypervirulence region of the Ti plasmid pTiBo542 (Jin et al., *J. Bacteriol.* **169**, 4417 (1987)) contained in a super-virulent *A. tumefaciens* A281 exhibiting extremely high transformation efficiency (Hood et al., *Biotechnol.* **2**, 702 (1984); Hood et al., *J. Bacteriol.* **168**, 1283 (1986); Komari et al., *J. Bacteriol.* **166**, 88 (1986); Jin et al., *J. Bacteriol.* **169**, 4417 (1987); Komari, *Plant Science* **60**, 223 (1987); ATCC Accession No. 37394.

Exemplary super-binary vectors known to those skilled in the art include pTOK162 (*see* Japanese Patent Appl. (Kokai) No. 4-222527, European Patent Applications EP 504,869 and EP 604,662, and United States Patent No. 5,591,616, herein incorporated by reference) and pTOK233 (*see* Komari, *Plant Cell Reports* **9**,303 (1990), and Ishida et al., *Nature Biotechnology* **14**, 745 (1996); herein incorporated by reference). Other super-binary vectors may be constructed by the methods set forth in the above references. Super-binary vector pTOK162 is capable of replication in both *E. coli* and in A. tumefaciens. Additionally, the vector contains the virB, virC and virG genes from the virulence region of pTiBo542. The plasmid also contains an antibiotic resistance gene, a selectable marker gene, and, if desired, a nucleic acid of interest to be transformed into the plant. Super-binary vectors of the invention can be constructed having the features described above for pTOK162. The T-region of the super-binary vectors and other vectors for use in the invention may be constructed to have restriction sites for the insertion of, for example, heterologous genes to be delivered to the monocot plant. Alternatively, heterologous nucleic acids to be transformed can be inserted in the T-DNA region of the vector by utilizing in vivo homologous recombination. *See*, Herrera-Esterella et al., *EMBO J.* **2**, 987 (1983); Horch et al., *Science* **223**, 496 (1984). Such homologous recombination relies on the fact that the super-binary vector has a region homologous with a region of pBR322 or other similar plasmids. Thus, when the two plasmids are brought together, a desired gene is

inserted into the super-binary vector by genetic recombination via the homologous regions.

Although binary vectors and specifically super-binary or supervirulent vectors are preferred in the practice of the present invention, other vectors known in the art (*i.e.*, integrating vectors) may also be used at the discretion of the skilled artisan.

Preferably, *Agrobacterium* vectors utilized in the methods of the present invention may be modified by recombinant nucleic acid techniques to contain a heterologous nucleic acid (*i.e.*, a gene or genes of interest) to be expressed in the transformed cells. The vectors of the present invention may also optionally comprise regulatory sequences useful or necessary for the transcription and translations of the heterologous nucleic acid. Such regulatory regions include but are not limited to transcriptional initiation regions (including promoters) operatively linked to the nucleic acid or gene of interest, 5′ leader sequences, terminator sequences, and other regulatory elements known to those skilled in the art. General molecular biology techniques that may be used to create the modified vectors used in the invention are well-known by those of skill in the art. *See, e.g.*, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (1989).

The term "heterologous" is used to indicate that a nucleic acid sequence (*i.e.*, a gene) or a protein has a different natural origin with respect to its current host. "Heterologous" is also used to indicate that one or more of the domains present in a protein differ in their natural origin with respect to other domains present. "Expression" refers to the transcription and translation of a structural heterologous nucleic acid to yield the encoded protein. The heterologous nucleic acid to be expressed is preferably incorporated into the T-region and is flanked by T-DNA border sequences of the *Agrobacterium* vector.

Any heterologous gene or nucleic acid that is desired to be expressed in a monocot plant is suitable for incorporation into the *Agrobacterium* vector of the present invention, and can be used in the methods of the invention. Heterologous genes to be transformed and expressed in the monocot plants of the present invention include but are not limited to genes that encode resistance to diseases, genes that encode resistance to insects, genes conferring nutritional value, genes conferring antifungal, antibacterial or antiviral activity, genes conferring resistance to herbicides, genes conferring improved plant or nutritional traits, and the like.

Alternatively, therapeutic (*e.g.*, for veterinary or medical uses) or immunogenic (*e.g.*, for vaccination) peptides and proteins can be expressed in monocots transformed by the methods of the present invention Likewise, the methods of the present invention may be used to transfer any nucleic acid for controlling gene expression into a monocot. For example, the nucleic acid to be transferred can encode an antisense oligonucleotide. Alternately, monocots can be transformed with one or more genes to reproduce enzymatic pathways for chemical synthesis or other industrial processes. Heterologous nucleic acids useful in the present invention may be naturally occurring and may be obtained from prokaryotes or eukaryotes (*e.g.*, bacteria, fungi, yeast, viruses, plants, insects, and mammals), or the nucleic acids may be synthesized in whole or in part.

Preferably, the *Agrobacterium* vectors used in the present invention will comprise a selectable marker gene for the selection of transformed cells. The selectable marker gene may be the only heterologous gene expressed by a transformed cell, or may be expressed in addition to another heterologous gene transformed into and expressed in the transformed cell. Selectable marker genes are utilized for the identification and selection of transformed cells or tissues. Selectable marker genes include genes encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (*NEO*) and hygromycin phosphotransferase (*HPT*), as well as genes conferring resistance to herbicidal compounds. Herbicide resistance genes generally code for a modified target protein insensitive to the herbicide or for an enzyme that degrades or detoxifies the herbicide in the plant before it can act. *See*, DeBlock et al., *EMBO J.* **6**, 2513 (1987); DeBlock et al., *Plant Physiol.* **91**, 691 (1989); Fromm et al., *BioTechnology* **8**, 833 (1990); Gordon-Kamm et al., *Plant Cell* **2**, 603 (1990). For example, resistance to glyphosphate or sulfonylurea herbicides has been obtained using genes coding for the mutant target enzymes, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and acetolactate synthase (ALS). Resistance to glufosinate ammonium, boromoxynil, and 2,4-dichlorophenoxyacetate (2,4-D) have been obtained by using bacterial genes encoding phosphinothricin acetyltransferase, a nitrilase, or a 2,4-dichlorophenoxyacetate monooxygenase, which detoxify the respective herbicides.

Selectable marker genes that can be useful in the practice of the present invention include, but are not limited to, genes encoding: neomycin

phosphotransferase II (Fraley et al., *CRC Critical Reviews in Plant Science* **4**, 1 (1986)); cyanamide hydratase (Maier-Greiner et al., *Proc. Natl. Acad. Sci. USA* **88**, 4250 (1991)); aspartate kinase; dihydrodipicolinate synthase (Perl et al., *BioTechnology* **11**, 715 (1993)); *bar* gene (Toki et al., *Plant Physiol.* **100**, 1503 (1992); Meagher et al., *Crop Sci.* **36**, 1367 (1996)); tryptophane decarboxylase (Goddijn et al., *Plant Mol. Biol.* **22**, 907 (1993)); neomycin phosphotransferase (*NEO*; Southern et al., *J. Mol. Appl. Gen.* **1**, 327 (1982)); hygromycin phosphotransferase (*HPT* or *HYG*; Shimizu et al., *Mol. Cell. Biol.* **6**, 1074 (1986)); dihydrofolate reductase (DHFR); phosphinothricin acetyltransferase (DeBlock et al., *EMBO J.* **6**, 2513 (1987)); 2,2- dichloropropionic acid dehalogenase (Buchanan-Wollatron et al., *J. Cell. Biochem.* **13D**, 330 (1989)); acetohydroxyacid synthase (United States Patent No. 4,761,373 to Anderson et al.; Haughn et al., *Mol. Gen. Genet.* **221**, 266 (1988)); 5-enolpyruvyl-shikimate-phosphate synthase (*aroA*; Comai et al., *Nature* **317**, 741 (1985)); haloarylnitrilase (WO 87/04181 to Stalker et al.); acetyl-coenzyme A carboxylase (Parker et al., *Plant Physiol.* **92**, 1220 (1990)); dihydropteroate synthase (*sulI*; Guerineau et al., *Plant Mol. Biol.* **15**, 127 (1990)); and 32 kDa photosystem II polypeptide (*psbA*; Hirschberg et al., *Science* **222**, 1346 (1983)).

Other genes that may be useful in the practice of the present invention include but are not limited to genes encoding resistance to: chloramphenicol (Herrera-Estrella et al., *EMBO J.* **2**, 987 (1983)); methotrexate (Herrera-Estrella et al., *Nature* **303**, 209 (1983); Meijer et al., *Plant Mol. Biol.* **16**, 807 (1991)); hygromycin (Waldron et al., *Plant Mol. Biol.* **5**, 103 (1985); Zhijian et al., *Plant Science* **108**, 219 (1995); Meijer et al., *Plant Mol. Bio.* **16**, 807 (1991)); streptomycin (Jones et al., *Mol. Gen. Genet.* **210**, 86 (1987)); spectinomycin (Bretagne- Sagnard et al., *Transgenic Res.* **5**, 131 (1996)); bleomycin (Hille et al., *Plant Mol. Biol.* **7**, 171 (1986)); sulfonamide (Guerineau et al., *Plant Mol. Bio.* **15**, 127 (1990); bromoxynil (Stalker et al., *Science* **242**, 419 (1988)); 2,4-D (Streber et al., *Bio/Technology* **7**, 811 (1989)); phosphinothricin (DeBlock et al., *EMBO J.* **6**, 2513 (1987)); spectinomycin (Bretagne-Sagnard and Chupeau, *Transgenic Research* **5**, 131 (1996)).

The *bar* gene confers herbicide resistance to glufosinate-type herbicides, such as phosphinothricin (PPT) or bialaphos, and the like, and can be used in the practice of the present invention. As noted above, other selectable markers that

could be used in the vector constructs include, but are not limited to, the *pat* gene, also for bialaphos and phosphinothricin resistance, the *ALS* gene for imidazolinone resistance, the *HPH* or *HYG* gene for hygromycin resistance, the EPSP synthase gene for glyphosate resistance, the *Hm1* gene for resistance to the Hc-toxin, and other selective agents used routinely and known to one of ordinary skill in the art. *See generally*, Yarranton, *Curr. Opin. Biotech.* **3**, 506 (1992); Chistopherson et al., *Proc. Natl. Acad. Sci. USA* **89**, 6314 (1992); Yao et al., *Cell* **71**, 63 (1992); Reznikoff, *Mol. Microbiol.* **6**, 2419 (1992); Barkley, et al., *The Operon* 177-220 (1980); Hu et al., *Cell* **48**, 555 (1987); Brown et al., *Cell* **49**, 603 (1987); Figge et al., *Cell* **52**, 713 (1988); Deuschle et al., *Proc. Natl. Acad. Sci. USA* **86**, 5400 (1989); Fuerst et al., *Proc. Natl. Acad. Sci. USA* **86**, 2549 (1989); Deuschle et al., *Science* **248**, 480 (1990); Labow et al., *Mol. Cell. Biol.* **10**, 3343 (1990); Zambretti et al., *Proc. Natl. Acad. Sci. USA* **89**, 3952 (1992); Baim et al., *Proc. Natl. Acad. Sci. USA* **88**, 5072 (1991); Wyborski et al., *Nuc. Acids Res.* **19**, 4647 (1991); Hillenand-Wissman, *Topics in Mol. And Struc. Biol.* **10**, 143 (1989); Degenkolb et al., *Antimicrob. Agents Chemother.* **35**, 1591 (1991); Kleinschnidt et al., *Biochemistry* **27**, 1094 (1988); Gatz et al., *Plant J.* **2**, 397 (1992); Gossen et al., *Proc. Natl. Acad. Sci. USA* **89**, 5547 (1992); Oliva et al., *Antimicrob. Agents Chemother.* **36**, 913 (1992); Hlavka et al., Handbook of Experimental Pharmacology **78**, (1985); and Gill et al., *Nature* **334**, 721 (1988). The disclosures described herein are incorporated by reference.

The above list of selectable marker genes are not meant to be limiting. Any selectable marker gene can be used in the present invention.

The concentration of *Agrobacterium* vector in the *Agrobacterium* solution of the present invention will vary according to the specific strain of *Agrobacterium* being used, as well as the species or strain of plant being transformed. For example, very high concentrations of *Agrobacterium* may damage the plant tissue to be transformed. Thus, the concentration of *Agrobacterium* useful in the methods of the invention may vary depending on the *Agrobacterium* strain utilized, the tissue being transformed, the monocot species being transformed, and the like. To optimize the transformation protocol for a particular monocot species or plant, the plant to be transformed may be incubated with various concentrations of *Agrobacterium*. Likewise, the level of marker gene expression and the transformation efficiency can be assessed for various *Agrobacterium*

concentrations. While the concentration of *Agrobacterium* may vary, generally a concentration range of about 1 x 10^3 cfu/ml to about 1 x 10^{10} cfu/ml can be employed, preferably a range of about 1 x 10^3 cfu/ml to about 1 x 10^9 cfu/ml, and more preferably a range of about 1 x 10^8 cfu/ml to about 1 x 10^9 cfu/ml.

Referring again to **FIG. 1**, after the monocot plant **80** growing in the first container **70** is placed in the vacuum chamber **10**, and the flowers or panicles **90** submerged in the *Agrobacterium* solution or suspension contained in the second container **100**, the vacuum chamber **10** is sealed, and a vacuum created in the chamber. The vacuum may be created by any of the known methods in the art. In one embodiment of the invention, vacuum connector **20** is connected to vacuum pump 110, which is used to create the vacuum within the chamber.

The term "vacuum" refers to an enclosed region of space (such as in a vacuum chamber) in which the pressure has been reduced below normal atmospheric pressure. The vacuum created is preferably such that the pressure within the chamber is lower than about 50 kiloPascals (kPascals), preferably is lower than 20 kPascals, more preferably is lower than about 10 kPascals, and most preferably is lower than about 5 kPascals, or even lower. A vacuum having a pressure of zero (0) kPascals would represent a nearly perfect vacuum. The vacuum is applied to the chamber for a period of time effective to cause the entry or entrance of the *Agrobacterium* solution or suspension containing one or more Agrobacterium clone(s) into the flower in contact with or submerged in the *Agrobacterium* solution or suspension. The term "cause the entrance of the *Agrobacterium* solution into the flower" means that a greater amount of the *Agrobacterium* solution enters the flower under vacuum conditions than would normally enter the flower in the absence of the vacuum.

The period of time required for the plant **80** to be subjected to the vacuum will vary according to the species of plant being used, the concentration of *Agrobacterium* in the *Agrobacterium* solution, the pressure maintained in the vacuum chamber **10**, and other factors. For example, this period of time may be for as little as 30 seconds, but is preferably for at least one minute, and is more preferably at for least 2 to 5 minutes, although keeping the plant **80** in the vacuum chamber **10** under vacuum conditions for longer periods of time than 5 minutes, and for shorter periods of time than 30 seconds, are clearly within the scope of the invention.

As used herein, the term "concurrently" means sufficiently close in time to produce a combined effect (that is, concurrently may be simultaneously, or it may be two or more events occurring within a short time period before or after each other). Accordingly, while the contacting of the flowers or panicles of the plant **80** with the *Agrobacterium* solution is said to occur concurrently with subjecting the plant **80** to a vacuum, it is clear that the contacting of the plant **80** with the *Agrobacterium* solution and the subjecting of the plant to a vacuum may occur simultaneously. However, it is also specifically intended that in one alternative embodiment of the invention, the flowers or panicles **90** of the plant **80** are soaked in the *Agrobacterium* solution for period of time prior to placing the plant **80** in the vacuum chamber **10**, after which the vacuum chamber **10** may be sealed and the vacuum created in the chamber. In this alternative embodiment, it is not necessary for the flowers or panicles **90** of the plant **80** to be in contact with the *Agrobacterium* during the time that the plant is subject to the vacuum. In such an embodiment, the second container **100** need not be present in the vacuum chamber **10** while the plant is being subjected to the vacuum. In this alternative embodiment, the flowers or panicles **90** may be pre-contacted (*i.e.*, pre-soaked or pre-submerged) in the *Agrobacterium* solution for a period of time that may be determined to be appropriate by the skilled artisan, prior to treatment with the vacuum. For example, the flowers or panicles **90** may be pre-contacted with the *Agrobacterium* solution for a period of time as short as 5 seconds, but preferably for longer than 30 seconds, and most preferably longer than a minute.

After the flowers or panicles **90** of the monocot plant **80** have been subjected to the vacuum for a sufficient amount of time to allow the *Agrobacterium* solution to enter the cells of the flower or panicles, then the vacuum is released from the vacuum chamber **10**. The plant **80** thus treated is then removed from the vacuum chamber **10**. If the plant **80** is growing in a first container **70**, the container **70** in which the plant **80** is growing is removed from the vacuum chamber **10**.

After removal from the vacuum chamber **10**, the plant may be subjected to optional resting and/or decontamination steps. In a decontamination step, the plants are contacted with or administered an antibiotic capable of inhibiting the growth of *Agrobacterium*. Antibiotics known in the art to inhibit *Agrobacterium* include cefotaxime, timetin, vancomycin, carbenicillin, and the like.

Concentrations of the antibiotic will vary according to what is standard for each antibiotic and may be optimized for a particular transformation protocol without undue experimentation by the skilled artisan. The antibiotic may be administered using methods of submersion, spraying, aerosol, and the like. The optional resting step can be performed in the absence of any selective pressures to permit recovery and proliferation of transformed cells. In the resting phase, plants are preferably allowed to rest in the dark or under subdued light, and most preferably in the absence of direct sunlight. The resting and decontamination steps may be carried out either together or in conjunction, in either order; alternatively one step may be carried out but not the other. Each optional step may be carried out for as long as is necessary to inhibit the growth of *Agrobacterium* (in the case of the decontamination step) or to increase the number of transformed cells (in the case of the resting step). Typically, the resting and decontamination steps (either individually or in conjunction) may be carried out for 1 to 7 days, preferably for 1 to 3 days, and more preferably for 1 to 2 days.

After the treated plant **80** is removed from the vacuum chamber **10**, and optionally subjected to a resting and/or decontamination phase, the treated plant **80** is grown (*i.e.*, is cultivated) to maturity according to methods appropriate to the specific species of plant, which methods are known to those skilled in the art. By "maturity" is meant that the plant **80** produces seeds. The seeds of the plant **80** may be collected by any suitable means known in the art.

The cultivated plants themselves and/or the seeds produced by the cultivated plants may be screened to determine if the plants (or certain cells of the plants) have been successfully transformed by the method of the present invention. "Screening" generally refers to identifying the cells exhibiting expression of a heterologous gene that has been transformed into the plant. Usually, screening is carried out to select successfully transformed seeds (*i.e.*, transgenic seeds) for further cultivation and plant generation (*i.e.*, for the production of transgenic plants). As mentioned above, in order to improve the ability to identify transformants, one may desire to employ a selectable or screenable marker gene as, or in addition to, the heterologous gene of interest. In this case, one could then generally assay the potentially transformed seeds or plants by exposing the seeds, plants, or seedlings to a selective agent or agents, or one could screen the seeds, plants or tissues of the plants for the desired marker gene. For example, transgenic

seeds may be screened under selective conditions, such as by growing the seeds or seedlings on media containing selective agents, such as antibiotics (*e.g.*, hygromycin, kanamycin, paromomycin or BASTA®), the successfully transformed plants having been transformed with genes encoding resistance to such selective agents.

To additionally confirm the presence of the heterologous nucleic acid or "transgene(s)" in the seeds of the cultivated plant **80** or in the regenerated plants produced from those seeds, a variety of assays may be performed. Such assays include, for example, molecular biological assays, such as Southern and Northern blotting and PCR; biochemical assays, such as detecting the presence of a protein product, *e.g.*, by immunological means (ELISAs and Western blots) or by enzymatic function; by plant part assays, such as leaf or root assays; and also, by analyzing the phenotype of the whole regenerated plant.

While Southern blotting and PCR may be used to detect the gene(s) in question, they do not provide information as to whether the gene is being expressed. Expression of the heterologous gene may be evaluated by specifically identifying the protein products of the introduced genes or by evaluating the phenotypic changes in the treated plant brought about by the expression of the genes introduced.

Assays for the production and identification of specific proteins may make use of physical-chemical, structural, functional, or other properties of the proteins. Unique physical-chemical or structural properties allow the proteins to be separated and identified by electrophoretic procedures, such as native or denaturing gel electrophoresis or isoelectric focusing, or by chromatographic techniques such as ion exchange or gel exclusion chromatography. The unique structures of individual proteins offer opportunities for use of specific antibodies to detect their presence in formats such as an ELISA assay. Combinations of approaches may be employed with even greater specificity such as Western blotting in which antibodies are used to locate individual gene products that have been separated by electrophoretic techniques. Additional techniques may be employed to absolutely confirm the identity of the product of interest such as evaluation by amino acid sequencing following purification. Although these techniques are among the most commonly employed, other procedures are known in the art and may be additionally used.

Apparatus useful in the practice of the present invention comprise a chamber of sufficient size to contain at least one monocot plant, which chamber is capable of being sealed and capable of containing an appropriate vacuum. Such a chamber may be a bell jar. Alternatively, the chamber may be a glass, plastic, or metal chamber comprising a top, bottom, and at least three walls (preferably four). The chamber may have an opening (i.e., a sealable door) that allows the ingress and egress of a plant or a container in which such a plant is growing. Apparatus useful in the practice of the invention also comprise means for generating a vacuum inside the container (vacuum generating means), a connector between the vacuum generating means and the chamber, and supporting means for affixing or supporting a plant within the chamber. Vacuum generating means are well known in the art and include vacuum pumps of many kinds. Suitable connectors between the vacuum generating means and the chamber are also known in the art and are generally tubes or pipes made of rubber or plastic materials. Supporting means may be designed to support or affix a container in which a plant is growing, but may also and alternatively comprise means for supporting or affixing a plant alone. Suitable examples of such supporting means are described above and are illustrated in **FIG. 1**. For example, referring to **FIG. 1**, the supporting means may be a pair of dowels **60**, the dowels being inserted into support holes/indentations **65**. Preferably the dowels **60** are removable and a series of in-line, parallel support holes/indentations **65** are located vertically along the back of the chamber such that the location of the dowels **60** (and thus, the distance of the plant or container from the bottom of the chamber) may be adjusted. Other supporting means include tying or binding means, racks or shelves located within the chamber, or magnetic means (i.e., a magnet on the bottom of a container in which a plant is growing may be contacted with a magnet located at the top of the chamber, thus supporting the container within the chamber). Other supporting means will be evident to those skilled in the art.

The following Examples are provided to illustrate the present invention, and should not be construed as limiting thereof. As used herein, the abbreviation ml means milliliters, min means minutes, g means grams, mg means milligrams, l or L means liters, rpm means revolutions per minute, h means hours, μM means

micromolar, cm means centimeters, OD means optical density, OD_{600} means optical density at 600 nm light wavelength, and M means molar.

EXAMPLE 1

Preparation of the *Agrobacterium tumefaciens* solution

5 ml of LB medium (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter, pH 7.0, autoclaved, with 25 mg/l kanamycin added) is inoculated with an *Agrobacterium tumifaciens* colony from a glycerol stock (strain LBA4404, containing the binary vector pocsGUSHyg, containing both a β-glucuronidase gene and a hygromycin resistance gene, each under the control of a constitutive promoter). The bacteria are grown at 28°C with shaking at 250 rpm, for 20 h, to stationary phase. The next day, 200 ml of LB medium in a 500 ml bottle are inoculated with 200 µl of the overnight culture and grown for 20 h to stationary phase at 28°C at 250 rpm. The bacterial culture is centrifuged at room temperature (RT) at 5500 rpm for 20 min and the supernatant is poured off. The bacterial pellet is resuspended in 500 ml of infiltration medium (5% sucrose, autoclaved, 300 uM acetosyringone and 0.05% Silwet L-77 added after autoclaving), yielding an *Agrobacterium* solution with an OD_{600} of approximately 0.8.

EXAMPLE 2

Vacuum infiltration of rice flowers

Rice plants of the varieties HUSAKU-SHIRAZU and SSANG DUO ZO with panicles three days before heading are selected for transformation. Panicles are carefully freed from the surrounding leaf sheaths, without cutting the leaves off, giving free access to the whole panicle. The whole plant, grown in soil in a 9x9 cm pot, is turned upside down and is fixed within a vacuum chamber such that the pot is affixed to the top of the chamber, with the plant directed towards the bottom of the chamber. 50 ml of the *Agrobacterium* solution described in Example 1 is filled into a 18 cm-test tube and the test tube is affixed in the vacuum chamber such that the freed rice panicles are immersed in the *Agrobacterium* solution.

The vacuum chamber is closed and a vacuum of 5 kPascal is applied to the chamber for two minutes. The vacuum is released and the plant is removed from the vacuum chamber. The treated panicles are covered with a paper bag in order to

maintain an appropriate humidity for the *Agrobacteria*. The plant is placed under conditions of no direct sunlight (*i.e.*, is rested) for one day. After one day, the paper bag is removed from the treated panicle and the plant is transferred to normal growing conditions and is cultivated until seeds have matured.

EXAMPLE 3
Screening for transgenic plants

The mature seeds of the treated panicles of Example 2 are harvested. The seed husks are removed and the seeds are surface sterilized (30 min at RT on a shaker in 6% calcium-hypochlorite containing 0.1 ml Triton X-100 per 100 ml, washed 4-5 times with sterile water) and germinated in water for 3 days. Transgenic seedlings are selected by transferring them onto metallic grids in containers, filled with water, each containing 20 mg per liter of Hygromycin B. The seedlings are grown under selective conditions for ten days, until a clear distinction between plants growing well under selective conditions and dying plants is observed. A one-centimeter piece of the second rice leaf is cut off from the surviving plants and is transferred to GUS staining solution (0.1 M K/Na-phosphate pH 7.0, 10 mM Na-EDTA, 0.1% Triton X-100, 5 mM Potassium ferricyanide (III), 5 mM Potassium ferrocyanide (II), 100 mg X-Gluc (5-Bromo-4-chloro-3-indolyl-beta-D-glucuronide acid cyclohexylammonium salt). A vacuum of 10 kPascal is applied for 5 minutes, after which the vacuum is released. The leaf pieces are further incubated in the staining solution at 37°C for 12 hours. Then, the leaves are screened for blue color development. The quality of blue staining, indicates that the plant has been successfully transformed. The successful transformation of the plant is then additionally confirmed by harvesting leaf material from the plants, isolating the genomic DNA from the plants, and probing the DNA for the transgene with the appropriate probe.

The foregoing is illustrative of the present invention and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

THAT WHICH IS CLAIMED IS:

1. An *in planta* method of transforming a monocotyledonous plant comprising:

contacting at least one flower of the monocotyledenous plant with a solution or suspension comprising an *Agrobacterium* clone; and

subjecting said plant to a vacuum effective to cause entry of the *Agrobacterium* clone into at least one flower of the plant.

2. A method according to Claim 1, wherein the monocotyledon plant is a rice plant.

3. A method according to Claim 2, wherein the rice plant is in the heading stage.

4. A method according to Claim 2, wherein the rice plant is in a developmental phase either prior to or after the heading stage.

5. A method according to Claim 2, wherein the rice plant is a Japonica or Indica variety.

6. A method according to Claim 1, wherein the contacting step and the step of subjecting the plant to a vacuum occur concurrently.

7. A method according to Claim 1, wherein the solution comprising *Agrobacterium* comprises a binary *Agrobacterium* vector.

8. A method according to Claim 1, wherein the solution comprising *Agrobacterium* comprises a supervirulent *Agrobacterium* vector.

9. A method according to Claim 1, wherein the *Agrobacterium* vector comprises a heterologous gene.

10. A method according to Claim 1, wherein the heterologous gene is selected from the group consisting of genes encoding resistance to antibiotics, genes encoding resistance to disease, genes encoding resistance to insects, genes encoding resistance to herbicides, and genes conferring improved plant or nutritional traits.

11. A method according to Claim 1, wherein the solution or suspension comprising *Agrobacterium* further comprises a wetting agent.

12. A method according to Claim 1, wherein the plant is subjected to the vacuum after the plant is placed inside a vacuum chamber.

13. A method according to Claim 12, wherein the contacting step occurs prior to placing the plant inside the vacuum chamber.

14. A method according to Claim 12, wherein the contacting step occurs while the plant is inside the vacuum chamber.

15. A method according to Claim 1, wherein the plant is subjected to the vacuum for a period of time of less than about five minutes.

16. A method according to Claim 1, wherein the plant is subjected to a resting step, and/or a decontamination step after being subjected to the vacuum.

17. A method of claim 1 wherein said solution or suspension comprises an agent which can induce the *vir* genes.

18. A method according to Claim 1, further comprising:
cultivating the plant to maturity.

19. A method according to Claim 18, further comprising:
collecting seeds from the plant after the plant has reached maturity.

20. An *in planta* method of transforming a rice plant comprising: contacting at least one panicle of the rice plant with a solution or suspension comprising at least one *Agrobacterium* clone; and

subjecting the rice plant to a vacuum effective to cause entry of the *Agrobacterium* clone into at least one flower of the panicle.

21. A method according to Claim 20, wherein the rice plant is in the heading phase.

22. A method according to Claim 20, wherein the rice plant is in a developmental phase either prior to or after the heading phase.

23. A method according to Claim 20, wherein the rice plant is a Japonica or Indica variety.

24. A method according to Claim 20, wherein the contacting step and the step of subjecting the rice plant to a vacuum occur concurrently.

25. A method according to Claim 20, wherein the solution comprising *Agrobacterium* comprises a binary *Agrobacterium* vector.

26. A method according to Claim 20, wherein the solution comprising *Agrobacterium* compresses a supervirulent *Agrobacterium* vector.

27. A method according to Claim 20, wherein the *Agrobacterium* vector comprises a heterologous gene.

28. A method according to Claim 20, wherein the heterologous gene is selected from the group consisting of genes encoding resistance to antibiotics, genes encoding resistance to disease, genes encoding resistance to insects, genes encoding resistance to herbicides, and genes conferring improved plant or nutritional traits.

29. A method according to Claim 20, wherein the solution comprising *Agrobacterium* further comprises a wetting agent.

30. A method according to Claim 20, wherein the rice plant is subjected to the vacuum after the rice plant is placed inside a vacuum chamber.

31. A method according to Claim 30, wherein the contacting step occurs prior to placing the rice plant inside the vacuum chamber.

32. A method according to Claim 30, wherein the contacting step occurs while the rice plant is inside the vacuum chamber.

33. A method according to Claim 20, wherein the rice plant is subjected to the vacuum for a period of time of less than about five minutes.

34. A method according to Claim 20, wherein the rice plant is subjected to a resting step, and/or a decontamination step after being subjected to the vacuum.

35. A method according to Claim 20, wherein said solution or suspension comprises an agent which can induce the *vir* genes.

36. A method according to Claim 20, further comprising:
cultivating the rice plant to maturity.

37. A method according to Claim 20, further comprising:
collecting seeds from the plant after the plant has reached maturity.

38. *An in planta* method of producing a transgenic rice plant, comprising:

contacting at least one panicle of a first rice plant with a solution comprising at least one *Agrobacterium* clone wherein the *Agrobacterium* clone comprises at least one heterologous gene;

subjecting the first rice plant to a vacuum effective to cause entry of the *Agrobacterium* clone into at least one flower of the panicle;

cultivating the first rice plant to maturity; and

collecting seeds of the first rice plant expressing the heterologous gene.

39. A method according to Claim 38, further comprising cultivating a mature, transgenic rice plant from the seeds of the first rice plant expressing the heterologous gene(s).

40. An apparatus for the transformation of a monocotyledenous plant, comprising:

a vacuum chamber of sufficient size to contain at least one monocotyledenous plant;

means for generating a vacuum;

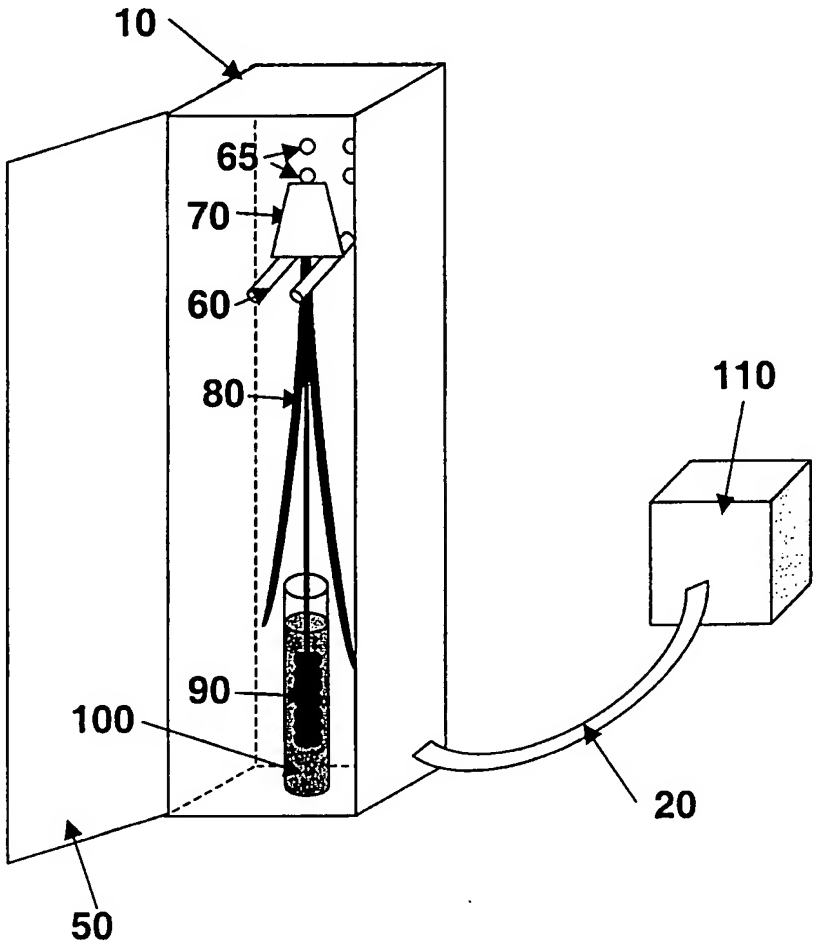
a connector that connects the means for generating a vacuum with the vacuum chamber; and

means for affixing the monocotyledenous plant inside the vacuum chamber.

41. An apparatus according to Claim 40, wherein the vacuum chamber is a bell jar.

42. An apparatus according to Claim 40, wherein the means for generating a vacuum is a vacuum pump.

Fig. 1



INTERNATIONAL SEARCH REPORT

Interr..tional Application No
PCT/US 00/22565

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 A01H1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KLOTI ET AL.: "Rice improvement by genetic transformation" MOL. BIOL. RICE (1998), 283-301. EDITOR(S): SHIMAMOTO, KO. PUBLISHER: SPRINGER-VERLAG TOKYO, TOKYO, JAPAN, XP000945319 See also Bechtold et al. (1993) cited below page 285, line 1 - line 4 --- -/--	1-39

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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Date of the actual completion of the international search	Date of mailing of the international search report
9 January 2001	16/01/2001

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/22565

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	BECHTOLD N ET AL: "IN PLANTA AGROBACTERIUM-MEDIATED TRANSFORMATION OF ADULT ARABIDOPSIS THALIANA PLANTS BY VACUUM INFILTRATION" METHODS IN MOLECULAR BIOLOGY,US,HUMANA PRESS INC., CLIFTON, NJ, vol. 82, 1998, pages 259-266, XP000914126 page 262, paragraph 3.3 -page 263	40-42
A	the whole document	1-39

Y	ROUT J R ET AL: "Agrobacterium -mediated gene transfer to rice (Oryza sativa L.);" IN VITRO,US,TISSUE CULTURE ASSOCIATION, vol. 31, no. 3, PART 02, 1995, page 28A XP002124030 ISSN: 0073-5655 the whole document	1-39

Y	BECHTOLD N ET AL: "IN PLANTA AGROBACTERIUM MEDIATED GENE TRANSFER BY INFILTRATION OF ADULT ARABIDOPSIS THALIANA PLANTS" COMPTES RENDUS DES SEANCES DE L'ACADEMIE DES SCIENCES. SERIE III: SCIENCES DE LA VIE,NL,ELSEVIER, AMSTERDAM, vol. 316, no. 10, 1993, pages 1194-1199, XP000907460 ISSN: 0764-4469 page 1196, column 2 page 1199, column 2	1-39

A	BENT: "Agrobacterium Vacuum Infiltration: Transformation Without Tissue Culture" IN VITRO, vol. 32, no. 3, 1996, page 32A XP000945347 the whole document	1-42

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